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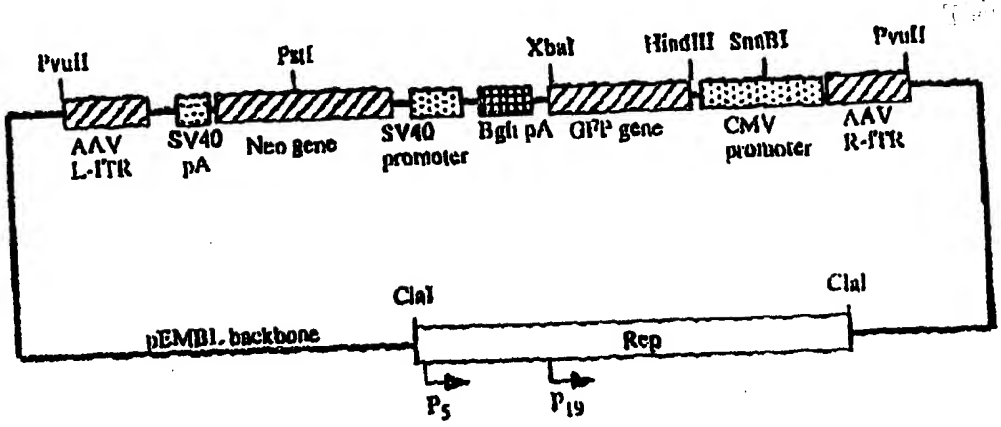

pITR(GFP-Neo)P$_5$Rep

(57) Abstract

The present invention refers to vectors derived form recombinant Adeno–associated virus (AVV) which comprise at least one selected transgene between the sequences of the 5' and 3' inverted terminal repeats (ITRs) from AAV, and a DNA sequence encoding one or more AAV Rep protein, or a fragment or a derivative thereof, outside of the context of the AAV ITRs. The vectors according to the invention are useful in gene therapy. The figure shows the diagram of a vector as per the invention, namely the plasmid pITR (GFP–Neo) P$_5$Rep.

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RECOMBINANT VECTORS DERIVED FROM ADENO-ASSOCIATED VIRUS SUITABLE FOR GENE THERAPY

DESCRIPTION

Field of the invention

The present invention relates to the field of vectors useful in (somatic) gene therapy and in the production thereof.

Background of the invention

The integration of therapeutic genes into specific locations of the DNA of nondividing cells accompanied by prolonged expression is the optimal strategy for somatic gene therapy. However, most currently available vector systems are not capable of both efficient transduction of non-dividing cells and long-term expression through stable integration of the vector genome into specific locations of the host cell DNA. Retroviral vectors based on Moloney leukaemia virus while capable of integration and stable long-term expression require cell division for efficient transduction. Additionally, the viruses integrate randomly into the host genome which may lead to insertional mutagenesis, or inadvertent activation of protooncogene expression due to promoter activity associated to retroviral LTR (long terminal repeats). In contrast, vectors based on adenovirus and herpes simplex virus are capable of transducing non-dividing cells but do not integrate into host cell DNA with appreciable frequency

Adeno-associated virus (AAV) has the unique capacity of preferentially integrating its viral DNA within a defined region of the cellular genome, thus reducing the risks of insertional mutagenesis associated with other viruses such as retroviruses that integrate at random positions. AAV is a nonpathogenic human parvovirus which usually requires Adenovirus (Ad) or herpesvirus as a helper to replicate efficiently. In the absence of helper virus the AAV genome integrates into host-cell genomic DNA at high frequency. Analysis of flanking sequences from latently infected cells have revealed integration of

the AAV genome into a specific locus in 60-70% of cases. The integration locus (aavs1) has been sequenced and localized to human chromosome $19_q13.3$-qter. Although AAV may replicate to a limited extent in the absence of helper virus in certain unusual conditions, as indicated above, the more common result is that infection of cells with AAV in the absence of helper functions results in the integration of AAV genome into the host cell chromosome. The integrated AAV genome can be rescued and replicated if cells containing an integrated provirus are superinfected with an helper virus such as Ad.

The AAV DNA genome is a linear single-stranded DNA molecule having a molecular weight of about $1.5x10^6$ daltons or approximately 4680 nucleotides long. The AAV2 genome has one copy of the 145 nucleotides long inverted terminal repeat (ITR) located at each end. The AAV ITR contain palindromic sequences that can fold over to form hairpin structures that function as primers during initiation of DNA replication. Additionally, the ITRs are needed for viral integration, rescue from the host genome, and encapsidation of viral nucleic acids into mature virions. Inserted in between the ITRs of AAV there is a unique region of about 4470 nucleotides that contains two main open reading frames (ORF). The right ORF encodes three capsid proteins VP1, VP2 and VP3. These three proteins form the viral particle and are produced from transcripts controlled by promoter P_{40} located at map position 40. The left open reading frame of the AAV genome encodes the rep gene. Two promoters located at map positions 5 and 19 (promoters P_5 and P_{19}, respectively) control the expression of the four polypeptides derived from this ORF. Rep proteins Rep 78 and Rep 68 are produced from the P_5 promoted transcripts, and Rep proteins Rep 52 and Rep 40 are synthesized from the P_{19} promoted transcripts. Targeting of integration may involve the AAV rep gene products. In particular, the larger polypeptides Rep 78 and Rep 69 have been shown to

bind in vitro the AAV ITR and the aavs1, and possess helicase and site-specific endonuclease activities which may be required for AAV replication as well as AAV integration.

The primary limitation of AAV for gene therapy is constituted by the packaging limit of the AAV virion that cannot exceed 4.5kb. The packaging size limitation would exclude several larger genes which may be considered as potential candidates for gene therapy programs. In order to overcome the above limitation, the remarkable ability of site-specific integration of AAV has been transferred to adenoviral vectors with larger packaging capacity by transferring the appropriate viral genes and cis-acting signals required for site-specific integration. For instance, in WO96/13598 is disclosed a hybrid Ad/AAV virus which has associated therewith a polycation sequence and the AAV rep gene ("hybrid virus conjugate" or "transinfection particle").

Description of the invention

The present invention exploits the AAV Rep gene and the AAV ITRs as part of a delivery system to achieve targeted integration of foreign DNA. Such targeted integration should provide a more effective and safer method of gene delivery.

The subject matter of the present invention is a recombinant DNA vector, characterized by the fact of comprising in combination the following nucleotide sequences:

(a) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) inverted terminal repeat (ITR) sequences;

(b) a DNA sequences encoding for one or more selected therapeutic genes of interest operatively linked to a constitutive or inducible promoter;

(c) DNA sequences of, or corresponding to, the 3'AAV ITR sequences;

(d) DNA sequence encoding an AAV Rep protein, or a

fragment or a derivative thereof, under the regulation of a constitutive or an inducible promoter;

(e) the gene encoding a selected protein of interest operatively linked to a constitutive or inducible promoter of point (b) above is inserted between the 5' and 3' ITRs from AAV; and

(f) the DNA sequence encoding one or more AAV Rep protein, or a fragment or a derivative thereof, under the regulation of a constitutive or inducible promoter, of point (d) above, is inserted outside of the context of the AAV ITRs.

The vectors according to the invention exploit the ability of the Rep proteins to mediate the excision of the AAV genome from a larger DNA context and subsequently to direct the site-specific integration of the ITR DNA fragment into the aavs1 site on chromosome 19.

Additionally, rep mediated integration into the aavs1 site should be limited to the DNA fragment enclosed within the 5' and 3' ITRs of AAV by virtue of the role of the ITR in targeting of the viral genome to the aavs1 site, and thus should allow the selective integration of the gene of interest with the concomitant exclusion of the remaining part of the DNA from the site specific integration process.

The consequence of this exclusion is that the integrated DNA can no longer be rescued in the form of replicating AAV even following superinfection with an helper virus such as Ad.

The present invention comprises the use of plasmides containing the Rep gene and the ITRs of AAV instead of recombinant AAV virions, thus it should permit the site-specific integration of DNA fragments of size larger than that normally carried by vectors based on the AAV genome.

According to a preferred embodiment of the present invention, the recombinant AAV vector is derived from plasmids pSub201 where the Cap gene has been deleted and the Rep gene is under the transcriptional control of the

P_5 and P_{19} However, additional promoters, such as α1 antitrypsin promoter, CMV immediate early promoter, SV_{40} early promoter, thymidine kinase promoter may be used for the same purpose.

Viral vectors constructed according to the present invention could be derived from adenovirus, herpes virus and baculovirus by insertion of the Rep gene and the ITRs of AAV as disclosed herein before. In particular an embodiment of the invention with viral vectors derived from Baculovirus can be found in the following examples 4 and 5.

The invention also refers to recombinant plasmids as disclosed herein before conjugated with liposomes, peptides, and DNA binding proteins that are used for gene transfer in vivo.

The therapeutic gene can be a human gene selected from the group comprising those encoding human Factor VIII coagulation factor, Factor IX coagulation factor, human LDL-receptor, human insulin, human distrophin or human CFTR.

The invention also refers to mammalian cell lines (in particular the cells are Keratinocytes) obtainable by transfection and/or infection of recombinant DNA vectors or recombinant viral particles according to the invention, as well as to pharmaceutical compositions comprising them and a pharmaceutical acceptable carrier.

Brief description of the drawings

Figure 1 shows the diagram of plasmid pITR(GFP-Neo).

Figure 2 shows the diagram of plasmid pITR(GFP-Neo)P_5Rep.

Figures 3A and 3B show reproduction of Southern blot of high molecular weight DNA from transfected HeLa cell clones, carried out using aavs1-specific and Neo-specific probes which reveal random integration of pITR(GFP-Neo).

Figures 4A and 4B show reproduction of Southern blot of high molecular weight DNA from transfected HeLa cell clones, carried out using aavs1-specific and Neo-specific

probes that shows site-specific integration of pITR(GFP-Neo) P_5Rep.

Figure 5 shows reproduction of Southern blot of high molecular weight DNA from HeLa cell clones transfected with plasmid pITR(GFP-Neo)P_5Rep, using Rep-specific probes.

Figure 6, shows the schematic representation of the recombinant baculoviruses used in this study. Baculovirus transfer plasmids were derived from pFastBac1 as described in Example 4. The E. Coli β-galactosidase gene (β-Gal), the hygromycin resistence gene (HYGRO), the Rep gene (Rep), and the AAV inverted terminal repeats (ITR) are indicated. The expression of β-Gal and of hygromycin resistence gene is driven by the CMV and TK promoter, respectively. The p5 and p19 promoters regulate expression of the Rep gene. Transcription initiation sites of the baculovirus polyhedrin promoter (pPolh), and of the p5 and p19 promoters are indicated by an arrow. Relevant EcoRV restriction sites are indicated with an E.

Figure 7 shows Baculovirus-mediated expression of β-Gal in MRC-5 and Huh-7 cells. 10^5 cells were plated and infected with Bac-β-Gal at the moi indicated. 48 hours p.i. total cell extracts were prepared, normalized for protein content, and assayed for β-Gal activity. Each column is the average of 2 independent assays, with the error bars representing SD.

Figure 8, shows Baculovirus-mediated expression of β -Gal activity in 293 cells infected with different constructs (Bac-ITR, Bac-ITR/RepS, Bac-ITR/RepA) at moi 50 and 100. Infection of 293 cells, preparation of cell extracts, and β-Gal assay, were carried out as indicated in Example 4.

Figure 9, shows the stability of recombinant Bac/AAV viruses. A) Southern blot analysis of Baculovirus genomes. One mg of viral DNA prepared from the third viral passage was digested with EcoRV, fractionated on 1% agarose gel, transferred to a nylon membrane and

hybridized with a probe specific for the transgene sequence. The expected bands of 4 and 10 kb are indicated by an arrow. B) The same filter was stripped and hybridized with a probe specific for the gentamicin gene which recognises a 1.3 kb band. The intensity of the bands indicated with arrowheads of both panels A and B were quantified by a phosphorimager apparatus according to manufacturer instructions. C) Western blot detection of Rep isoforms expressed in sf9 cells infected with different baculoviruses at an moi of 10. Two days p.i. total cell extracts were analysed for the presence of Rep proteins. Rep 78, Rep 68, and Rep 52 are indicated by an arrow.

Figure 10 shows Southern blot analysis of 293 hygr clones derived from Bac-ITR infection. Ten micrograms of genomic DNA from each clone was digested with ApaI, fractionated on 1% agarose gel, and transferred to a nylon membrane. A) Hybrydization to an aavs1 probe. The two detected bands of roughly 2.5 and 2.8 kb correspond to the aavs1 preintegration site. B) Same membrane after rehybridization to a transgene specific probe. Position of molecular weight standards (in kilobases) is indicated.

Figure 11 shows Southern blot analysis of 293 hyg.r derived from Bac-ITR/RepA infection. Conditions of infection and DNA analysis are as described in Fig. 10. A) Hybridization to aavs1 probe. B) Same membrane hybridized to transgene probe. The upshifted bands that are annealed to both probes are considered to be indicative of site-specific integration and are indicated with an arrowhead. Position of molecular weight standards (in kilobases) is indicated.

Figure 12 shows ITR-aavs1 junction sequences from Bac-ITR/RepA infected 293 and MRC-5 cells. Common bases at ITR-aavs1 junction are underlined. Sequences not belonging to ITR or aavs1 are bolded. X62488 was identified in the GeneBank with a Blast search. Sequence

are depicted in ITR, aavs1 order.

Figure 13 shows a schematic representation of the recombinant anenovirus Ad LBITR-GFP/Hy used in this study.

Figure 14 shows PCR amplification of aasv1-AAV-ITR junction. (A) Southern blot analysis of amplification products obtained using as template DNA extracted from infected cells. Total DNA was isolated from Huh7 cells infected with AdLBITR-GFP/Hy (moi=1 pfu/cell) (lane 2) and in combination with HdRS1032 (lane 3) or HdRA1034 (lane 4) (moi=1 bfu/cell). DNA from mock infected cells is in lane 1. A nested PCR was set up using two sets of primers. The first amplification was performed with primers 16s and 15a while for the second reaction a nested set of primers, Cr2 shift (GATAGACCAGACCTGAGCTATGGGAG) and 17s long (TTAACTACAAGGAACCCCTAGTGATGG) was chosen. 1/10 of the PCR reaction was loaded on 1.2 % agarose gel in duplicate, transferred on nylon membrane and hybridized with two different probes derived from aavs1 or AAV-ITR DNAs.

(B) Amplified DNA was cloned and sequenced. The sequences which have been reported represent the most frequent clones obtained in two different experiments performed in Huh7 and HepG2 cell lines.

Deposits

The strain Escherichia coli K12 containing the plasmid pITR(GFP-Neo)P_5Rep according to the invention has been deposited on December 5, 1996 with "The National Collections of Industrial and Marine Bacteria" (NCIMB), Aberdeen, Scotland, UK. The above strain was given the access number NCIMB 40832.

So far, a general description has been given of the present invention. With the aid of the following examples, a more detailed description will now be given of specific embodiments thereof, with the purpose of giving a clearer understanding of objects, features, advantages and methods of application of the invention.

The examples are illustrative, and do not limit the scope of the present invention.

Example 1

Construction of plasmids pITR(GFP-Neo) and pITR(GFP-Neo)P5rep

Plasmid pSub201 (R.J. Samulski et al., J. Virol., 1987) was cut with Xba I to remove the Rep and Cap coding sequences of adeno-associated virus (AAV) and the 4.0 Kb DNA fragment containing the inverted terminal repeats (ITR) of AAV, was then ligated to a 1.7 kb DNA fragment carrying a Nhe I restriction site at either end. This fragment contains the cDNA of the Green fluorescent protein (GFP) (R.J. Samulski et al., J. Virol., 1987) flanked at its 5'-end by the CMV immediate early promoter and enhancer and by the Bovine growth hormone (BGH) polyadenylation signal at its 3'-end. The GFP fragment was obtained by PCR amplification with sequence specific primers using as template a derivative of vector pCDNA-3 (Invitrogen) in which the GFP cDNA had been cloned [pCD3(GFP)]. The primer specific for the BGH polyadenylation signal was designed so that it contains a Sac II site located near the Nhe I site at the 3'-end. The construct so obtained was named pITR(GFP). Plasmid pITR(GFP-Neo) was produced by inserting the blunted EcoRI/BamHI fragment from pRc/RSV (Invitrogen) which contains the SV40 early promoter, neomycin resistance gene, and SV40 polyadenylation signal into the blunted Sac II site of pITR(GFP).

pITR(GFP-Neo)P5Rep was derived from PCR amplification of nucleotides 138-2234 of the AAV genome with sequence specific primers using plasmid pTAV-2 (R. Heilbronn et al., J. Virol., 1990) as template. The amplified DNA fragment was digested with Cla I and cloned into the Cla I site of plasmid pITR(GFP-Neo).

Example 2

Tranfection of recombinant plasmids

Plasmids pITR(GFP-Neo)(Fig. 1) and pITR(GFP-

Neo)P_5Rep (Fig.2), as already said, carried gene for the green fluorescenc eprotein (GFP) under the transcriptional control of the CMV early promoter, and thye neomycin resistance gene under the control of the SV40 early promoter. These two genes have been inserted between the 5' and 3' ITRs of AAV. Additionally, plasmid pITR(GFP-Neo)P_5Rep carries the Rep gene under the transcriptional control of the P_5 and P_{19} promoters cloned into the Cla I site of the plasmid vector and thus it is located outside the two ITRs of AAV.

The efficiency of site-specific integration of these two constructs was assessed by calcium phosphate transfection of HeLa cells.

Cell transfections

HeLa cells were maintained in Dulbeccos's modified Eagle's medium DMEM supplemented with 10% Fetal Calf serum (FCS), 2mM gilutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in 10 cm dishes (Falcon) at 37°C in 5% CO_2. Stock HeLa cells were routinely passaged every three days by treatment with trypsin (0,05%) and EDTA (0.53mM) and replated at cell densities appropriate for exponential growth. 16 hours prior to transfection, cells were seeded at a density of $1x10^6$ cells in 10 cm plates, and incubated for 12 hours at 37° C in 5% Co_2. The medium was then replaced and cells were then incubated for additional 4 hours at 37°C. Twenty micrograms of plasmid DNA (10 micrograms of the indicated plasmid plus 10 micrograms of carrier DNA) were precipitated in calcium phosphate using the calcium phosphate mammalian cell transfection kit (5prime-3prime) following the manufacturer's instructions. The DNA precipitate was added directly to each plate in a 1 ml volume. Sixteen hours posttransfection the medium was replaced and the cells were incubated for additional 32 hours. The transfected cells were then treated with trypsin and EDTA and the cell suspension derived from a single 10 cm plate was diluted 1 to 3 in selection medium

(DMEM 10% FCS, 2mM glutamine, 100 units/ml penicillin, 100µg/ml streptomycin, and 800µg/ml G418) and plated on three 15 cm plates (Falcon). Neomycin resistant clones were isolated after ten days of selection, expanded, and processed for genomic DNA extraction and Southern blot analysis.

Southern blot analysis of neomycin resistant clones

HeLa cell clones resistant to neomycin were grown in 10 cm plates and the cell monolayer was detached by scraping and washed twice with 2 ml of PBS. The cell suspension was transferred to Eppendorf tubes, spun for 10 min at 3000 rpm at 4°C in a Eppendorf microfuge, and the cell pellet was resuspended in 0.5 ml TEN (50 mM Tris-Cl, pH7.5, 150 mM NaCl, 10mM EDTA). SDS and proteinase K were added to each sample to final concentrations of 1% and 1mg/ml, respectively. The cell lysate was incubated for 4 hours at 56°C and subjected to three consecutive extractions with Phenol (equilibrated with 10 mM Tris-Cl, pH 8.0, 1mM EDTA) Phenol and Chlorophorm (1:1 ration), and Chlorophorm. The aqueous phase was precipitated with two volumes of ETOH after addition of sodium acetate solution (pH 6.0) to a final contration of 0.3M. The DNA pellet was washed once with 0.5 ml 70% ETOH, resuspended in 0.5 ml of H_2O, and incubated overnight at 4°C. Ten micrograms of high molecular weight chromosomal DNA was incubated with 40 units of the restriction enzyme BamHI (New England Biolabs) in a 0.1 ml volume for 12 hours at 37°C. The digested DNA was electrophoresed on a 0.8% agarose gel, transferred to nylon membrane (Hybond™-N+; Amersham) as recommended by the manufacturer and hybridized overnight at 65°C in Church buffer (7% SDS, 0.25 M NaPi, pH 7.2, 1mM EDTA pH 8.0, 0.1 g/ml BSA) with random primed ^{32}P-labeled probes. Approximately $2x10^6$ cpm/ml were used in each hybridization. Filters were washed in 40 mM NaPi, pH 7.2, 1mM EDTA, pH 8.0, 1% SDS. at 65°C three times for 20 min., and then in 0.1xSSC, 0.1% SDS at 65°C for 20 min.

Filters were exposed to X-ray film with an intensifying screen overnight. To determine site-specific integration of the ITR DNA fragment, filters were first hybridized with a probe specific for the neomycin gene, the hybridized probe was then removed by boiling the filters in 0.2 x SSC, 1% SDS for 10 min, the same filters were then hybridized to a probe specific for the aavs1 site.

Example 3

Analysis of transfected clones

Rearrangements of the aavs1 site resulting from the integration of the transfected plasmid were assessed by comparing the hybridization pattern of genomic blots using aavs1-specific and neo-gene specific probes to those of mock transfected cells DNA. The probe specific for the chromosome 19 was obtained by random priming reaction using as template a DNA fragment derived from plasmid pRVK (K. Berns, Cornell Medical School, New York, N.Y.) covering nucleotides 1-3525 of aavs1. The neo specific probe was derived from a random priming reaction with a 630 bp DNA fragment as template that includes most of the ORF of the neomycin resistance gene. The site-specific integration of the transfected plasmid was scored when: i) The hybridization pattern of the genomic DNA using the aavs1 probe is different from that of the mock transfected cells DNA in that it contains an additional fragment recognized by the aavs1 probe; ii) The same additional fragment revealed with the genomic probe is also detected with the neo specific probe.

The analysis of HeLa cell clones derived from the transfection of plasmid pITR(GFP-Neo) is represented in Figure 3. The genomic DNA extracted from 11 independent clones was digested with restriction enzyme BamHI and the DNA was resolved on an agarose gel. Southern blot hybridazion of these DNAs with a ^{32}P-labeled probe specific for the aavs1 site shows that the probe hybridized to two fragments of approximately 3.5 and 2.3 kb in size. Additionally, the hybridization pattern of

the transfected clones DNA is identical to that of the mock transfected cells DNA (Fig. 3A). In contrast, hybridization with a ^{32}P-labeled probe specific for the neomycin gene clearly shows that all these clones have been transfected with plasmid carrying the resistance marker since the neo specific probe hybridizes to a single band in each lane ranging in size between 8 and 20 kb, but no hybridization of this probe to the mock transfected cells DNA is detectable (Fig. 3B). Thus, in all cases analyzed (39 individual clones) the integration of the neo gene has occurred at sites other than the aavs1.

Transfection of plasmid pITR(GFP-Neo)P_5Rep resulted in a distinctly different hybridization pattern. As shown in Figure 4, hybridization with a aavs1-specific probe shows that in 25% of the clones analyzed (8 clones out of 32) an additional fragment ranging in size between 5 and 10 kb is hybridized by the genomic probe. In Figure 4 most of the clones wityh this type of hybridization pattern are shown, and the indicative of site specific integration is indicated with an arrowhead. This fragment is not present in digested DNA of other transfected clones and neither in the mock transfected cells DNA where only the two bands of 3.5 and 2.3 kb can be detected. Most importantly, the same fragment is hybridized by the probe specific for the aavs 1 site, indicating that the neomycin gene has been inserted into the aavs1 site (Fig. 4B). The data presented here clearly indicates that integration of the transfected plasmid DNA into the aavs1 site occurs with high efficiency and is dependent on the expression of the Rep gene.

The selective integration of the ITR DNA fragment was assessed by performing a Southern blot on the genomic DNA of the clones transfected with plasmid pITR(GFP-Neo)P_5Rep using a probe specific for the Rep gene (nt 138 to 2234 of AAV genome). As shown in Fig. 5, the probe hybridizes to several DNA bands in each lane. However,

the DNA fragment hybridized by the rep probe does not match in size the fragment revealed by both the neo and aavs1 probes. Thus, it is possible to conclude that although part of the plasmid pITR(GFP-Neo)P_5Rep may have integrated randomly in the host chromosomes upon transfection into HeLa cells, in most cases no insertion of the DNA sequences located outside of the ITR region has occurred into the aavs1 site.

Example 4:

Baculovirus can transiently transduce primary human and rat hepatocytes as well as a subset of stable cell lines. To prolong transgene expression we have developed new hybrid vectors which associate key elements from adeno-associated virus (AAV) with the elevated transducing capacity of baculovirus.

Vector constructs.

Figure 6 shows the vectors used in this study.

To determine the transduction efficiency of the recombinant baculoviruses and to establish stable cell clones from the infected cells, the β-gal and hygromicin resistence (hyg^r) genes were inserted between the AAV ITRs and cloned downstream of the baculovirus polyheidrin promoter (pPolh) (Bac-ITR). Additionally, the AAV Rep gene under the control of its own promoters p5 and p19 was cloned outside of the ITRs either in the sense orientation (Bac-ITR/RepS) or in the antisense orientation (Bac-ITR/RepA) with respect to the pPolh promoter. Lastly, baculoviruses carrying the CMV-β-gal expression cassette (Bac-β-Gal) or the Rep gene (Bac-Rep) were also constructed as control of transduction efficiency and Rep expression.

Recombinant baculoviruses were constructed using the transfer vector pFastBac1 (pFB1) (Gibco-BRL). A β-gal expressing cassette was derived from plasmid pCMV-b (Clontech). The EcoR I-Hind III fragment containing the CMV promoter, the β-gal gene, and the SV40 polyadenylation signal was inserted in the modified sites

of pFB1 generating plasmid pFB1Bac/CMV-b. To construct the chimeric Bac/AAV vectors, a p5-Rep-poly(A) cassette (ClaI, SpeI) was subcloned in the NspV, SpeI sites of pFB1 generating pFB1Rep-A.

A transgene cassette was assembled in plasmid pLitmus-28 (New England Biolabs) by three cloning steps generating pLit/AAV-hyg-β-gal which contains the following sequences: (i) the AAV ITRs derived from plasmid pSub201 (Samulski, R. J., L.-S. Chang, and T. Shenk. 1987.) as a Pvu II fragment and subcloned in the blunted Xba I site of pLitmus-28; (ii) the hygromycin resistance gene excised from plasmid pCEP-4 (Invitrogen) by digestion with Nru I and Not I and inserted between the ITRs in the filled-in Xba I site; (iii) the β-gal cassette derived from plasmid CMV-β which was introduced between the ITRs by blunt-end cloning into the filled-in Not I and Hind III sites.

Plasmid pLit/AAV-hyg-β-gal was digested with Spe I and Avr II and the 6.5 kb fragment containing the transgene cassette was subcloned in the modified pFB1 vector (digested with Xba I and Avr II) or pFB1RepA to generate Bac/ITR and Bac/ITR-RepA, respectively. Similarly, to generate plasmid Bac-RepS, the p5-Rep fragment was inserted in the Sfu I site of pFB1. Bac-ITR/RepS was derived from Bac-Rep-S by introducing the transgene cassette, as Avr II-Spe I fragment, in the Spe I site.

Recombinant baculoviruses were produced according to manufacter's instructions (GIBCO-BRL). Viruses were propagated in sf9 insect cells according to standard methods (O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1992).

Budded virus from insect cell culture medium was filtered on 0.22 mm filter and concentrated by ultrafiltration in a 45Ti rotor (30000 rpm; 75 min). The viral pellet was resuspended in phosphate saline buffer (PBS) and titered by plaque assay on sf9 insect cells.

Viruses were produced at high titer (ranging from $1x10^9$ to $9x10^9$ pfu/ml) and used for infection of mammalian cells.

Cell culture.

293, Huh-7, and MRC-5 cells were maintained in Dulbecco's modified Eagle's medium DMEM supplemented with 10% Fetal Calf serum (FCS), 2mM glutamine, 100 units/ml penicillin, and 100 mg/ ml streptomycin. Cells were grown in 10 cm dishes (Falcon) at 37^0 C in 5% CO2. Stock cells were routinely passaged every three days by treatment with trypsin (0.05%) and EDTA (0.53 mM) and replated at cell densities appropriate for exponential growth.

To isolate stable cell clones, the infected cells from a single 6 cm plate were plated at a density of 2x104 cells per plate in 10 plates containing selection medium (DMEM 10% FCS and 300 mg/ml hygromicin). Hygromicin resistant clones were isolated after ten days of selection, expanded, and processed for genomic DNA extraction, Southern blot, and FISH analysis. Assays for β-gal activity were carried out using the β-galactosidase enzyme assay sistem (Promega) as described by the manufacturer. β-gal was detected histochemically in cells fixed with 0.5 % glutaraldhyde solution in PBS and by incubation for 4 hours with staining solution (4mM K4[Fe(CN)6], 4mM K3[Fe(CN)6], 40 mM MgCl2, 0.4 mg/ml X-gal in PBS).

In an initial functional analysis of the transducing capacity of the recombinant baculovirus vectors, we observed that the transient β-gal expression in infected 293 cells generally increased as a function of the multiplicity of infection (moi). To determine whether the transducing capacity of baculovirus could be extended to other cell types, baculovirus transduction efficiency in human diploid fibroblasts MRC-5 was compared to that observed in human hepatoma cell line Huh-7 (fig 7). The hepatoma cell line has been shown to be extremely susceptible to baculovirus transduction (Hofmann, C., V.

Sandig, G. Jennings, M. Rudolph, P. Schlag, and M. Strauss. 1995.)

These two cell lines showed comparable levels of β-gal, thus indicating that these cells are equally susceptible to baculovirus infection, and similar results were obtained upon infection of primary rat fibroblasts. These results indicate that baculovirus transducing capacity can also be extended to primary cultures that are not necessarily of hepatic origin.

Southern blot analysis.

When the transduction efficiency of Bac-ITR, Bac-ITR/RepA, and Bac-ITR/RepS was compared by infecting 293 cells at a moi of 50 and 100, we noticed that it was the Bac-ITR virus was significantly better than Bac-ITR/RepA (fig 8). However, both viruses displayed a similar increase in β-gal expression as a function of moi. In contrast, very little, if any, β-gal expression could be detected upon infection of 293 cells with Bac-ITR/RepS.

To determine whether the Bac-ITR/RepS genome had undergone partial rearrangement during virus amplification, thus explaining its low transducing efficiency, we examined the integrity of Bac/AAV genomes by Southern blot. Genomic DNA was digested with EcoR V, and resolved on a agarose gel. EcoR V was chosen because it cleaves once within the ITR-flanked transgene sequence and once within the gentamicin gene, releasing three fragments of 10, 4, and 1.3 kb, respectively (fig 9A).

Baculovirus genomic DNA was prepared from 1 ml of the third viral passage according to standard protocols (O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1992). One mg of viral DNA was digested with EcoRV which releases two fragments of 4 and 10 kb. Following electrophoresis in 1% agarose gel, the digested fragments were transferred onto a nylon membrane (HybondN+) and processed according to manufacter's instructions and hybridized overnight at 65oC in Church buffer (7% SDS, 0.25 M NaPi, pH 7.2, 1 mM EDTA, pH 8.0, 0.1 g/ml BSA)

with random primed 32P-labeled probes. The following probes were used: for the gentamicin gene a 1.3 kb EcoRV, BamHI fragment derived from plasmid pFastBac1, and for the transgene cassette a 6.5 kb Avr II-Spe I fragment derived from plasmid pLit/AAV-hyg-β-gal.

The gentamicin gene is present in all virus genomes and therefore was used as an internal control (fig 9B). Quantification of transgene probe hybridization, normalized for that of gentamicin, indicated that with Bac-ITR/RepA it was 11.4 fold higher than with Bac-ITR/RepS, whereas no significant difference was observed between Bac-ITR/RepA and Bac/ITR. These data suggest that the low transduction efficiency of Bac-ITR/RepS may be ascribed to the loss or rearrangement of the ITR-flanked transgene cassette during the process of baculovirus amplification.

The three recombinant viral genomes produced in E. coli did not show any detectable rearrangement, therefore we hypothesized that loss of the β-gal and hygr expression cassette occurred during Bac-ITR/RepS amplification in insect cells.

Western blot analysis.

In view of the role of the Rep polypeptides in promoting the selective excision and amplification of the ITR-flanked DNA, we speculated that loss of the transgene cassette could be associated to the expression of Rep isoforms in sf9 cells. To verify this hypothesis, insect cells were infected with the Bac/AAV vectors and the Rep expression pattern was assessed by Western blot using a polyclonal antibody which recognizes all four Rep isoforms. 2x 10^6 sf9 cells were infected at a moi of 10, and 72 hours later cells were collected, washed in PBS, and lysed by freezing and thawing three times in lysis buffer [10 mM Hepes buffer pH.8.0, 0.6 M NaCl]. 200 mg of proteins of each sample were separated by SDS-12.5% PAGE, and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was immersed in 1% skimmed

milk in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) (blocking buffer) for 20 min at room temperature. The anti-Rep polyclonal antiserum diluted in blocking buffer was applied to the nitrocellulose membrane and incubated for 1 h at room temperature. The membrane was then washed repeatedly with blocking buffer. The bands were visualized with ECL reagents according to manufacter's instructions (Amersham).

Rep 78, 68, and 52 could be clearly detected in Bac-Rep infected cells, whereas no Rep polypeptides could be detected in Bac-ITR cell lysates (fig 9C). The expression level of Rep polypeptides differed between Bac-ITR/RepS and Bac-ITR/RepA cell lysates. Rep expression was lower in cells infected with the Bac/AAV carrying the Rep gene in the antisense orientation with respect to pPolh, as compared to the corresponding virus carrying the Rep gene in a sense orientation. In agreement with the Southern blot analysis of genomic DNA, these results suggest that the ITR-flanked cassette is destabilized in Bac/AAV virus genome when Rep proteins are expressed above a certain threshold.

Bac/AAV infection of 293 cells.

Recent studies have shown that transfection of 293 cells with a plasmid carrying the Rep gene and a Green fluorescence protein (GFP) expression cassette inserted between the AAV terminal repeats results in an highly efficient integration of the ITR-flanked DNA (Balagué, C., M. Kalla, and W. W. Zhang. 1997, Surosky, R. T., M. Urabe, S. G. Godwin, S. A. McQuiston, G. J. Kurtzman, K. Ozawa, and G. Natsoulis. 1997). On the basis of this observation we wanted to determine whether the delivery of AAV components mediated by baculovirus can establish more stable cell clones. To this end, 293 cells were infected with Bac-β-Gal, Bac-ITR/RepA, and Bac-ITR vectors and the transduction efficiency of these viruses and stable integration of the ITR-flanked DNA was compared by measuring β-gal expression and the production

of hygr clones.

The reporter activity measured in infected cells cultured in absence of selection as a function of time (up to 14 days p.i.). Although the transduction efficiency determined at day 1 p.i. was similar with all three vectors, the residual β-gal activity at day 14 p. i., was much higher in the Bac-ITR/RepA infected cells than either those infected with Bac-ITR, which do not contain Rep gene, or Bac-β-Gal, which contains only the β-gal expression cassette.

To assess the influence of the AAV components on the frequency of integration events, a sample of cells infected with either Bac-ITR or Bac-ITR/RepA was collected at day 4 p. i. and plated in hygromycin selection medium. Infection of 293 cells with Bac-ITR/RepA results in a significantly higher number of stable cell clones as compared to cells infected with Bac ITR. The increase in number of hygr clones ranged in several experiments from 10 to 50 fold. Five hundred hygr clones were obtained by plating 20,000 cells infected with Bac-ITR/RepA. On the basis of the cloning efficiency of the 293 cells (50%) and on the percentage of the cells transduced by Bac-ITR/RepA (50%), we estimate that approximately 10% of the transduced cells carrying the hygromicin gene stably integrated in the host genome. In addition, the majority of the hygr clones (>90%) expressed β-gal (determined by histochemical staining of the infected cells), suggesting that the entire ITR-flanked transgene cassette had been inserted into the host chromosome. These results thus indicate that both under selective and nonselective conditions, Baculovirus delivery of the Rep gene allows a very efficient transduction of ITR-flanked cassette, probably by mediating its insertion into the human genome.

Site -specific Integration

To verify the efficiency of site specific integration mediated by the Bac/AAV vectors, hyg^r clones

from 293 cells infected with Bac-ITR/RepA and Bac-ITR were isolated, expanded, and total genomic DNA was subjected to Southern blot analysis. Genomic DNA was prepared as previously described and processed as described above. The rearrangment of the aavs1 site resulting from the integration of the ITR-flanked transgene cassette was assessed using aavs1 and hygro-β-gal specific probes. Filters were first hybridized with a probe specific for the transgene cassette, the hybridized probe was then removed by boiling the filters in 0.2 x SSC, 1% SDS for 10 min, and the same filters were then hybridized to a probe covering nucleotides 1-3525 of aavs1. Site-specific integration of the transduced transgene cassette was scored when the aavs1 specific probe recognized additional bands absent in mock infected cells, and when the same additional bands were also detected by the transgene probe.

The analysis of some of the 293 clones derived from infection with Bac-ITR is shown in figure 10. The genomic DNA extracted from 9 indipendent clones was digested with Apa I, and analysed by Southern blot. Apa I was chosen because it cleaves once in the aavs1 sequence but does not cleave within the transgene. Thus, the insertion of the transduced DNA into the aavs1 site should be easily detectable by the altered pattern of one of the aavs1 derived restriction fragments. The aavs1 specific probe recognizes two bands of approximately 2.5 and 2.8 kb (Fig. 10A, lanes 2 to 10). Additionally, the hybridization pattern of the infected clones DNA is identical to that of the mock infected cells DNA (Fig. 10A, lane 1). Single or double bands ranging in size from 8 to 20 kb were detected in selected clones DNA when the same genomic blot was hybridized with a probe specific for the transgene cassette. Thus, in all clones analysed no obvious rearrangement of the aavs1 site could be ascribed to the infection of Bac-ITR, indicating that the integration of the transgene occurred at sites other than

aavs1.

Figure 11 shows the hybridization pattern obtained with the clones derived from the Bac-ITR/RepA infected cells. In 13 out of 22 clones (59%) (Fig. 11A, clones C1, D1, B1, H1, G4, E1, A2, H3, F3, H4, C3, D2, and D4) rearrangement of the aavs1 region was apparent. The same genomic blot was then hybridized with a transgene probe to ascertain the presence of the transgene sequences within the rearranged aavs1 bands. In 8 out of 13 clones (61%) (Fig. 11B, clones C1, B1, H1 G4, E1, C3, D2, D4) bands were detected which hybridized to the transgene probe matching those obtained with aavs1 probe (shown with an arrow), indicating that the transgene was indeed inserted in the aavs1 site. Thus, site-specific integration occurred in 8 out of 22 clones (36%) analysed. Additional transgene bands detected only with the transgene probe were present in half the clones analyzed. These bands might be due to rearrangements in the aavs1 region flanking the transgene sequences (Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook D. E. Housman, N. Epstein, and L. A. Hunter. 1991.) or to multiple insertions at sites other than aavs1.

Fluorescence in situ hybridization (FISH).

To confirm the site-specific integration of the transduced ITR-DNA cassette, and to differentiate between single and multiple integration events into different sites, FISH analysis of metaphase spreads was performed on infected 293 cells using aavs1 and transgene specific probes. Metaphases were scored as positive only if both probes were colocalized on both sister chromatids of a given chromosome. A 6.5 kb DNA fragment corresponding to the transgene cassette and a 80 kb aavs1 DNA fragment isolated by screening a genomic DNA library were labelled using the Nick Translation Kit (Boehringer Mannheim) according to manufacturer's instructions and used as probe in chromosome analysis.

The chromosome spreads from selected clones were

prepared according to typical cytogenetic techniques (Lawrence, J. B., C. A. Villnave, and R. H. Singer. 1988.). Cytogenetic preparations were pretreated with pepsin solution and dehydrated through cold 70, 90, and 100% ethanol. The preparations were then denatured using a 50% formamide solution. For each sample, 200 ng of probe, 2 mg of human Cot-1 DNA, 9 mg of sonicated salmon sperm DNA were precipitated and resuspended in hybridization buffer (50% formamide, 2X SSC, 1% BSA, and 10% Dextran sulfate). Probes were denatured for 8 min at 80°C and subsequently incubated for 10 min at 37°C to allow preannealing of repeated sequences. Finally, the hybridization solution was placed on the samples, covered with coverslips and incubated overnight at 37 °C in a moist chamber. The samples were then washed three times in 50% formamide and three times in 2x SSC at 42 °C. Visualization of the biotin-labeled probe was carried out by repeated incubations with Cy3-avidin (Amersham), biotinylated anti-avidin D (vector Laboratories), and Cy3-avidin. The digoxigenin-labeled probe was detected using mouse anti-digoxigenin antibody, digoxigenin-labeled anti-mouse, and FITC-labeled anti-digoxigenin antibodies (Boehringer Mannheim). Alternatively, FITC-avidin (Vector Laboratories) and rhodamine labeled antidigoxigenin (Boehringer Mannheim) were used. After immunodetection, slides were counterstained with 200 ng/ml of 4', 6'-diamidino-2-phenylindole (DAPI). Ultraviolet excitation was used to locate metaphases and photographic images were taken by a CCD camera (Photometrics) using green (FITC signal) or blue violet (Cy3 or rhodamine) illumination. Images were processed using Adobe Photoshop on an Apple Quadra computer.

Four hundred hyg^r clones were pooled 10 days after infection with Bac-ITR/RepA and analyzed by FISH. The transgene probe colocalized with the aavs1 probe in 10 out of 24 (41%) metaphases analyzed, whereas in the remaining methaphases the transgene probe was located on

different chromosomes that were not detected by the aavs1 probe. The aavs1 probe annealed to three or four chromosomes 19 in most of metaphases analyzed, in agreement with the polyploid nature of this cell line. No hybridization of the transgene probe was detected on mock infected cells. Although FISH analysis is not strictly quantitative, the site-specific integration frequency of 41% established by FISH analysis of pooled cell clones is in good agreement with the integration frequency of 36% derived from Southern blot analysis of single clones. The colocalization of the transgene and aavs1 probes was also observed upon FISH analysis of metaphases derived from the individual clones that had been previously characterized by Southern blot.

PCR amplification of the ITR-aavs1 junction.

To precisely identify the integration site of the transgene cassette, 293 and MRC-5 cells were infected with Bac-ITR/RepA and the genomic DNA was extracted and subjected to amplification of the ITR-aavs1 junction. For this purpose, two sets of nested primers specific for the AAV terminal repeat and chromosome 19 were used (Goodman S., X. Xiao, R. E. Donahue, A. Moulton, J. Miller, C. Walsh., N. S. Young. R. J. Samulski, and A. W. Nienhuis. 1994). As control, genomic DNA from cells infected with Bac-ITR was also analyzed.

Primers 16s (AAV) 5'-GTAGCATGGCGGGTTAATCA and 15a (aavs1) 5'-GCGCGCATAAGCCAGTAGAGC were used in the first round of PCR amplification using 0.5 mg of genomic DNA as substrate. After an initial incubation for 4 minute at 94° C, the reaction mixture was subjected to 30 cycles of PCR amplification with the following parameters: 1 minute 94° C, 1 minute 55° C, 2 minute 72° C. One percent of the amplification product was diluted into a new reaction mixture containing a set of nested primers with the following sequences: 17s (AAV) 5'-TTAACTACAAGGAACCCCTA, and Cr2 (aavs1) 5'-ACAATGGCCAGGGCCAGGCAG. The PCR parameters were the same as for the first amplification.

For molecular cloning of the amplified junction fragments, the product of the second round of amplification was purified on a 1% agarose gel, and subcloned by blunt end ligation into plasmid pZERO-2.1 (Invitrogen). Sequencing was performed using standard chain termination protocols.

Specific DNA bands were amplified from MRC-5 cells infected with Bac-ITR/RepA at a moi of 100, and 500. In contrast, no specific product was detected with mock- or Bac-ITR infected cells. A similar protocol was utilized to amplify the ITR-aavs1 junction from Bac-ITR/RepA infected 293 cells. The amplified DNA bands were cloned and sequenced (fig 12). In MRC-5 cells, the insertion of the ITR-flanked transgene cassette was mapped at nt 1111 of aavs1 where a short homology between the AAV ITR and aavs1 can be identified in the GCC triplet. A deletion of 43 and 83 bases within the ITR sequence was also identified. Similarly, the ITR-aavs1 junctions amplified from pooled hyg^r 293 clones indicated that the insertion of the transgene cassette had occurred at nt 1012, 1080, and 1096 of aavs1. In clone 293-2 an insertion of three nucleotides was detected at the juction between the ITR and aavs1. A larger insert of 101 nucleotides was identified in clone 293-1. This insert is partially homologous to sequence X62488 deposited in the GeneBank data base by Samulsky and coworkers, and is derived from amplification of ITR-aavs1 junction from AAV infected cells (Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook D. E. Housman, N. Epstein, and L. A. Hunter. 1991.). These results indicate that, Baculovirus mediated transduction of human cells results in the integration of the ITR-flanked DNA cassette in a specific region of the aavs1 site.

Example 5

Construction of a first generation Adenoviral vector carrying the integration cassette.

Preparation of the plasmid pLBITR-GFP/Hy:

a) The AAV2-ITRGFP/HygroR cassette was constructed inserting the humanized version of green fluorescent protein (GFP) gene driven by HCMV promoter from the pGreen Lantern plasmid (GIBCO BRL) and the Hygromicin B resistance gene fused to the Tk promoter between AAV2 ITRs in the contest of pLITMUS28 plasmid generating pITRGFP/Hygro.

b) The AAV2-ITRGFP/Hygro cassette was excised from pITRGFP/Hygro by XbaI-BglII digestion and cloned into pABS.4 digested XbaI-BamHI generating pABITR-GFP/Hygro.

c) Finally, the ITR-GFP/Hygro cassette obtained from pABITR-GFP/Hygro by PacI digestion, was inserted in the unique PacI restriction site of an Ad5 genome deleted of the E1 and E3 regions and fully infectious when transfected in 293 cells, to generate the plasmid pLBITR-GFP/Hy.

Preparation of the recombinant adenovirus Ad LBITR-GFP/Hy

a) 60 mm dishes of semiconfluent monolayer of 293 cells were transfected with 3 μg of pLBITR-GFP/Hy using a standard calcium-phosphate technique (Sambrook,J. et al.1989).

b) The cell monolayer was incubated overnight at 37 C, the medium was removed and 10 ml of medium-agarose overlay was added.

c) Plaques were visible after 10 days of incubation at 37 C. Picking, screening and amplification of the isolated plaques were performed as described (Hitt,M. et al. 1995).

Analysis of viral DNA.

Purification of viral DNA from infected cell and purified virions was performed by digestion at 37 C in proteinaseK/SDS buffer (10mM Tris-HCl, pH7.5, 1mM EDTA/ 1% SDS/1 mg/ml proteinaseK) followed by phenol extraction and ethanol precipitation. Episomal DNA was isolated from chromosomal DNA following the Hirt protocol.

Construction of Adenovirus Helper Dependent vectors carrying the AAV2Rep gene.

Plasmids were constructed using standard protocols (Sambrook,J. et al.1989).

Derivation of the plasmid pRP1030:

pRP1030 was derived from pRP1001 (Parks, R.J. et al. 1996) by deleting all Ad5 coding sequence and substituting it with a lambda phage DNA stuffer.

Derivation of the plasmid pRS1032:

a) pABT7RepDATG:

The helper dependent plasmid pRS1032 was derived from pRP1030. It contains the insertion of a cassette constituted by Rep78 ATG deletion mutant fused to T7 promoter. Rep 78 gene was obtained as described in M. Horer et al (1995), by mutating the ATG start codon for Rep 52/40 to GGA (methionine>glycine, aminoacid 225) and the G of the splice donor site for expression of spliced versions Rep 68/40 (nucleotide 1907) to A. Rep 78DATG was obtained deleting by PCR the first ATG of Rep open reading frame.

Derivation of the plasmid pRA1034:

The helper dependent plasmid pRA1034 was derived from pRP1030. pRA1034 contains Rep 78 gene fused to Δ 137a1antitrypsin promoter.

Rescue and amplifications of Ad Helper-Dependent vectors.

a) Semiconfluent monolayers of 293cre cells (Parks, R.J. et al. 1996) in 60 mm dishes were infected with AdLC8cLuc or AdLC8CARP1.2.at a multiplicity of infection (m.o.i.) of 5 plaque-forming units (pfu)/cell.

b) 4 hr after infection 293cre cells were transfected using standard calcium-phosphate technique with 5 mg of helper-dependent vector (pRS1032, pRS1033, pRA1034) for 6 hr at 37 C,

c) the medium was replaced and the cells incubated until the monolayer showed complete cytopathic effect.

d) The cells were scraped into the medium and the

virus released by freezing and thawing.

e) The resulting crude lysate was serially passaged on 60-mm dishes of 293cre cells.

f) During each round of amplification of the three different helper-dependent vectors, the 293cre monolayers were infected with AdLC8cLuc or AdLC8CARP1.2 at an m.o.i. of 1 and incubated at 37 C for 4 hr before the infection with 1/10 of the crude lysate.

Site -specific Integration of AAV-ITR transgene

It has been reported that Rep expressed from a cassette located outside viral ITRs can acts in trans promoting site-specific integration of an AAV-ITRs-flanked transgene (Balangue' J.Virol 1997, Surosky, J.Virol. 1997).

Figure 14 demonstrates that Rep 78 expressed from HdRA1034 mediates integration of the rescued transgene in aavs1 locus.

HepG2 and HuH7 cells have been coinfected with HdRA1034 and Ad LBITR-GFP/Hy, a first generation Ad vector carrying AAV-ITRs flanking green fluorescent protein and Hygromicin B resistance (fig. 13)
48 hours post-infection cells have been harvested and genomic DNA has been extracted. A nested PCR-based assay on genomic DNA was set up to detect junctions between aavs1 and viral ITRs. The nested PCR was set up using two sets of primers. The first amplification was performed with primers 16s and 15a while for the second reaction a nested set of primers, Cr2 shift (GATAGACCAGACCTGAGCTATGGGAG) and 17s long (TTAACTACAAGGAACCCCTAGTGATGG) was chosen. 1/10 of the PCR reaction was loaded on 1.2 % agarose gel in duplicate, transferred on nylon membrane and hybridized with two different probes derived from aavs1 or AAV-ITR DNAs.

aavs1 primer sequences was chosen in the region located downstream the 100 nucleotide aavs1 region identified as preferential sites for site specific integration (Samulski R.J. et al. EMBO J. 1991).

Amplified DNA has been loaded on agarose gel in duplicate and analyzed by Southern blot using aavs1 and AAVITR specific probes. Figure 14A shows the result obtained infecting Huh7 cells. Similar results were observed infecting Hep G2 cell line. Positive signals has been detected only in cells infected with Rep expressing virus. No signal was present in cell infected with HdRA1032 carrying the Rep78DATG mutant or with AdLBGITR-GFP/Hy alone. Probing the Southern blots with either AAV-ITR or aavs1 radiolabelled DNA, the hybridization pattern appeared to be identical. Three major bands spanning 100-500 nucleotides superimposed on a smear has been detected. The result suggested that integration events occured in cluster in at least three different regions upstream the aavs1 oligonucleotides annealing site.

Amplified DNA was eluted from gel, cloned and sequenced. As preliminary report about junction sequencing, the sequence of the most frequent junctions observed in Huh7 and HepG2 has been shown in figure 1B. Although AAV-ITRs of our transgene derive from pSUB201, therefore are in flop orientation, we got clones in which the AAV-ITRs are integrated in flip orientation. Terminal sequence heterogeneity imply that AAV-ITRs flanked transgene was replicated before integration (Linden et al. 1996).

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CLAIMS

1. A recombinant DNA vector, characterized by the fact of comprising in combination the following nucleotide sequences:

(a) DNA sequences of, or corresponding to, the 5' adeno-associated virus (AAV) inverted terminal repeat (ITR) sequences;

(b) a DNA sequences encoding for one or more selected therapeutic genes of interest operatively linked to a constitutive or inducible promoter;

(c) DNA sequences of, or corresponding to, the 3'AAV ITR sequences;

(d) DNA sequence encoding an AAV Rep protein, or a fragment or a derivative thereof, under the regulation of a constitutive or an inducible promoter;

(e) the gene encoding a selected protein of interest operatively linked to a constitutive or inducible promoter of point (b) above is inserted between the 5' and 3' ITRs from AAV; and

(f) the DNA sequence encoding one or more AAV Rep protein, or a fragment or a derivative thereof, under the regulation of a constitutive or inducible promoter, of point (d) above, is inserted outside of the context of the AAV ITRs.

2. A recombinant viral particle, characterized by the fact of carrying the recombinant DNA vector sequences according to claim 1.

3. The recombinant viral particle of claim 2 carrying the recombinant DNA vector sequences according to claim 1, wherein the virus vector is derived from adenovirus.

4. The recombinant viral particle of claim 2 carrying the recombinant DNA vector sequences according to claim 1, wherein the virus vector is derived from herpes virus.

5. The recombinant viral particle of claim 2 carrying the recombinant DNA vector sequences according

to claim 1, wherein the virus vector is derived from baculovirus.

6. The recombinant DNA vector according to claim 1, wherein the vector is conjugated with liposomes that are used for gene transfer in vivo.

7. The recombinant DNA vector according to claim 1, wherein the vector is conjugated with peptides that are used for gene transfer in vivo.

8. The recombinant DNA vector according to claim 1, wherein the vector is conjugated with DNA binding proteins that are used for gene transfer in vivo

9. The recombinant particles according to claims 1 to 8, wherein the Cap gene has been deleted and the Rep gene is under the transcriptional control of the $AAVP_5$ and P_{19} promoters or under the transcriptional control of the other promoters such as CMV immediate early promoter, SV_{40} early promoter, thymidine kinase promoter, α1 anti-trypsin promoter.

10. The recombinant particles according to claims 1 to 9, wherein the therapeutic gene is a human gene to be used for the therapy of a genetic or of a metabolic disorder.

11. The recombinant particles according to claim 10, wherein the therapeutic gene is chosen among those encoding human Factor VIII coagulation factor, Factor IX coagulation factor, human LDL-receptor, human insulin, human distrophin or human CFTR.

12. Mammalian cell lines, characterized by the fact that they are obtainable by transfection and or infection with particles according to claims 1 to 9.

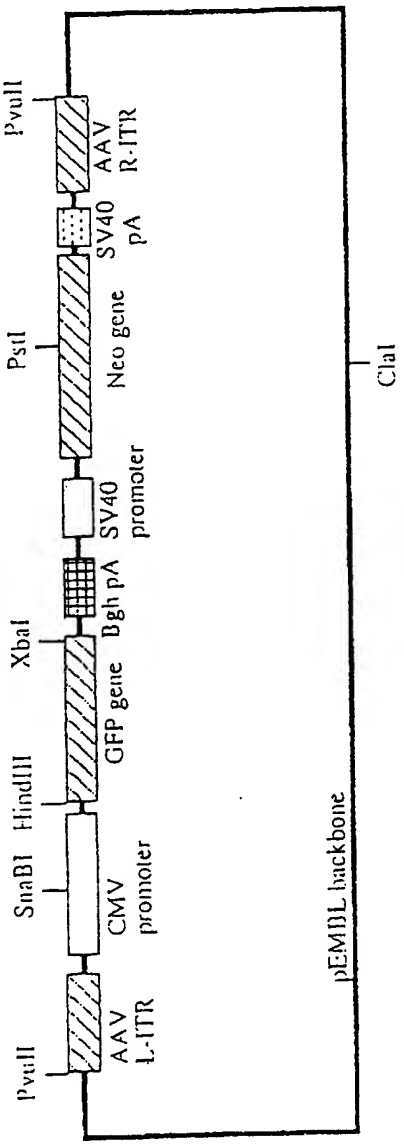
13. Mammalian cell lines, characterized by the fact that they are obtainable by transfection and or infection with particles according to claim 10 wherein the cells are keratinocytes.

14. Mammalian cell lines, characterized by the fact that they are obtainable by transfection and or infection with particles according to claim 10 wherein the cells

are hematopoietic stem cells.

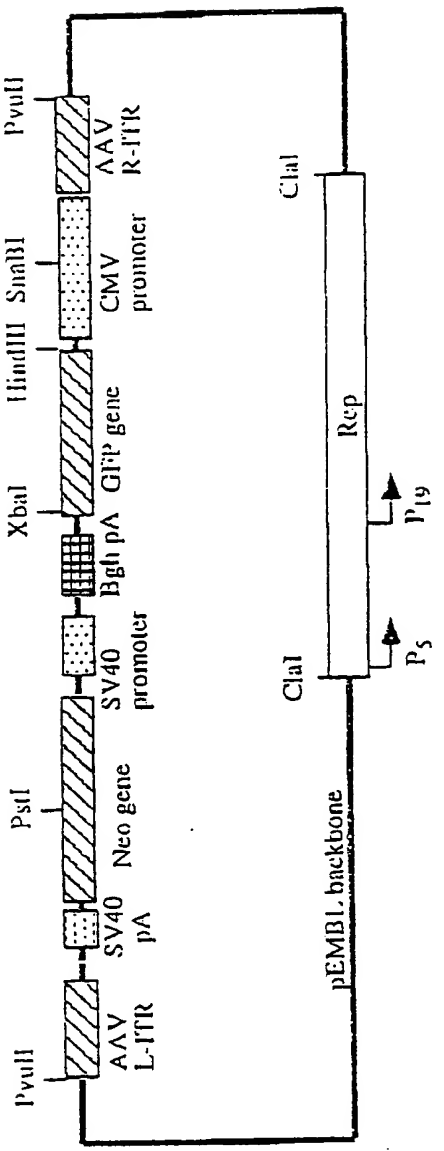
15. A pharmaceutical composition, characterized by the fact of comprising the recombinant viral particles of claims 2 to 5 as the active principle and a pharmaceutically acceptable carrier.

16. A pharmaceutical composition, characterized by the fact of comprising the recombinant DNA vector according to claim 6 to 8 as the active principle and a pharmaceutically acceptable carrier.



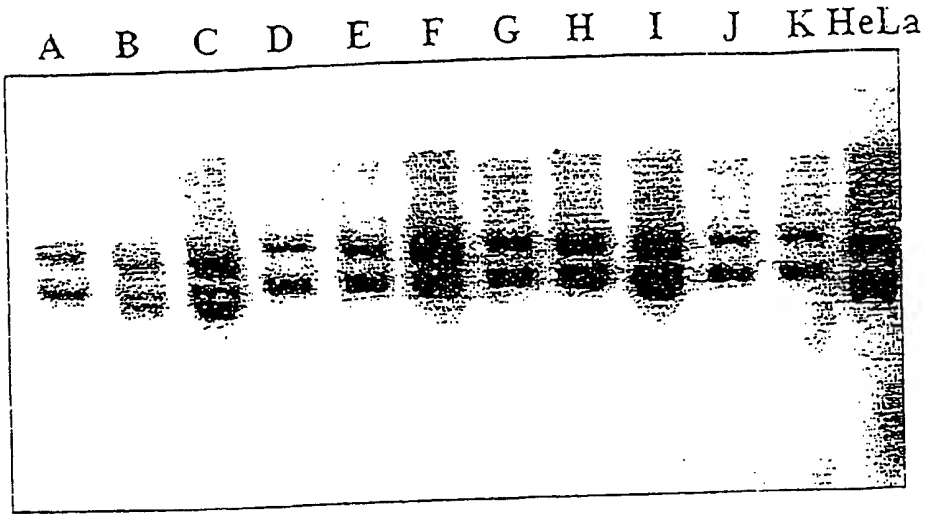
pITR(GFP-Neo)

FIG. 1



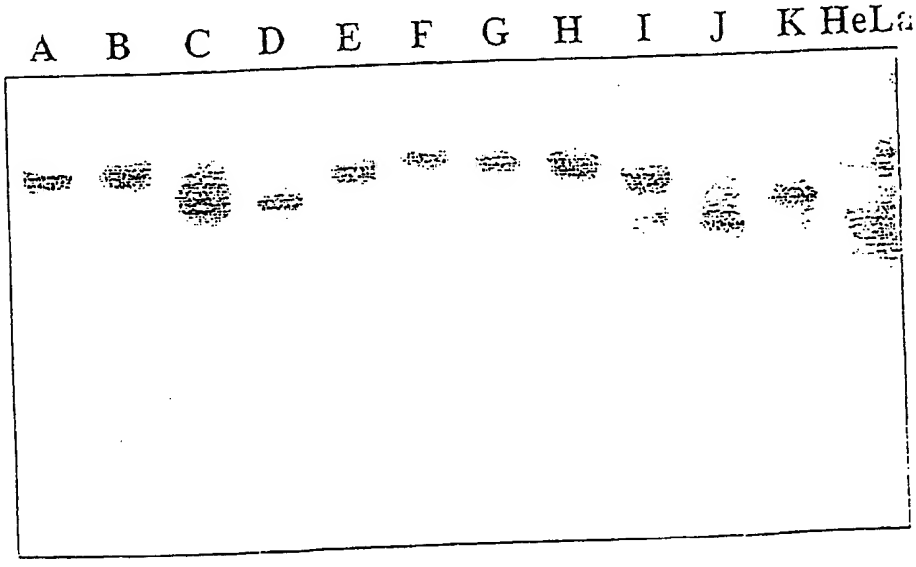
pITR(GFP-Neo)P5Rep

FIG. 2



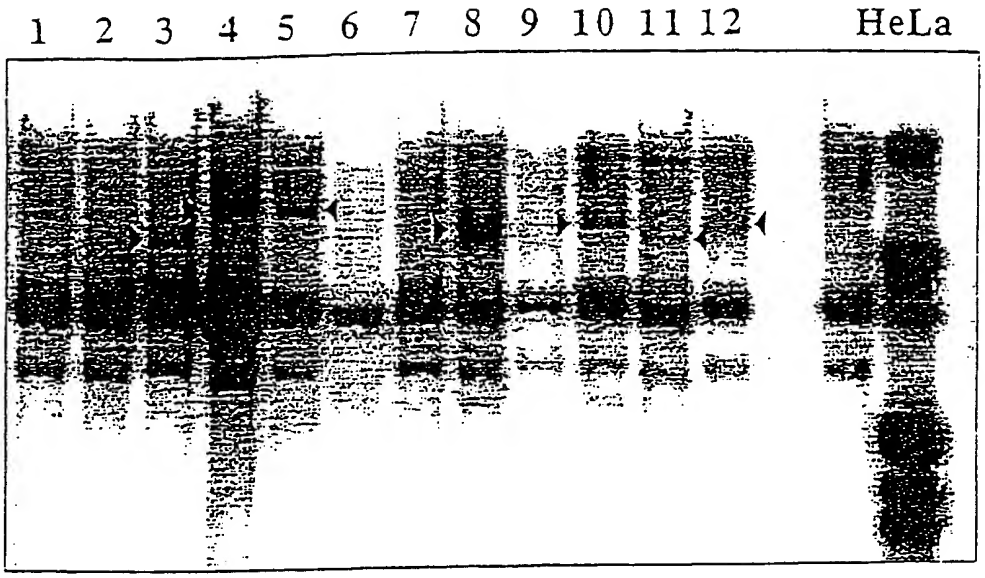
AAVS1 probe

FIG. 3A

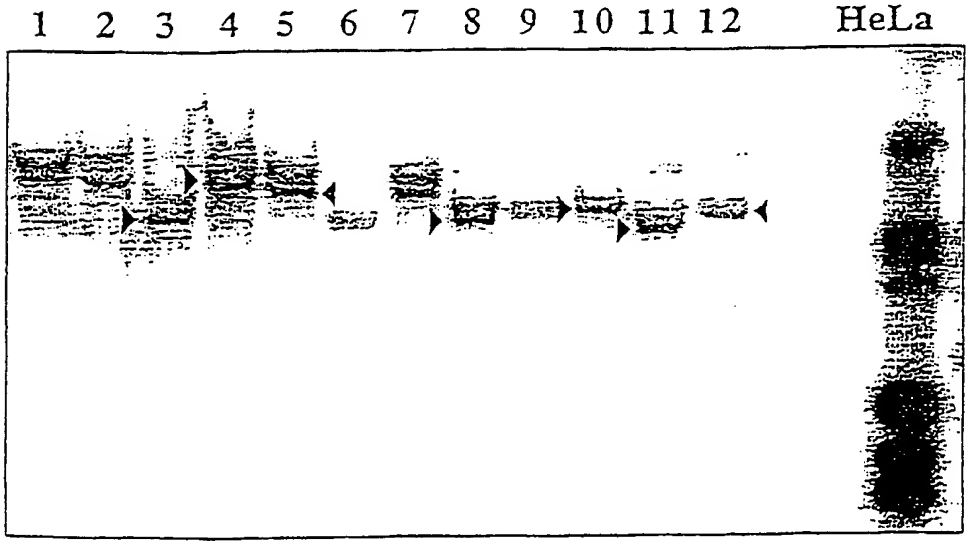


Neo probe

FIG. 3B



AAVS1 probe FIG. 4A



Neo probe FIG. 4B

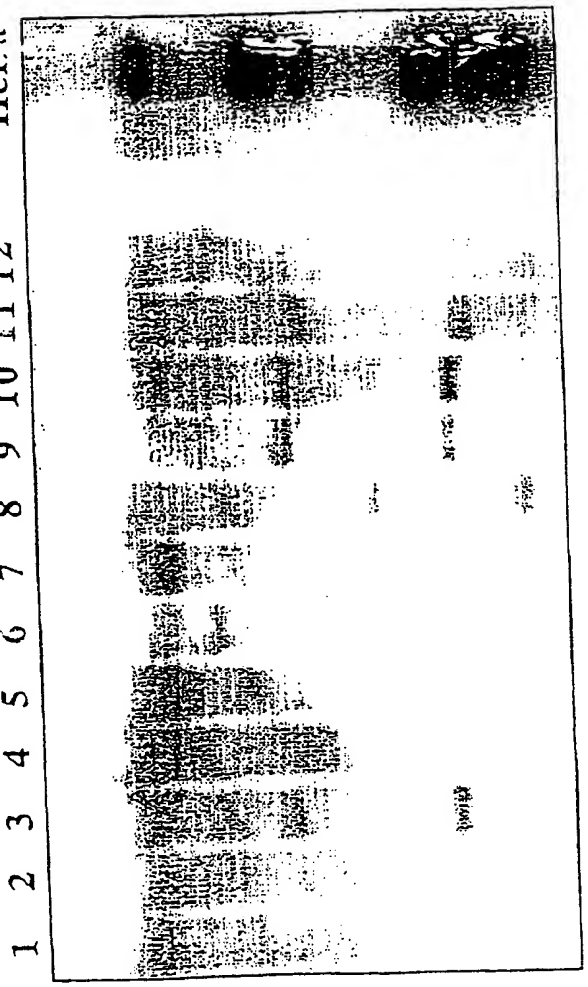


FIG. 5

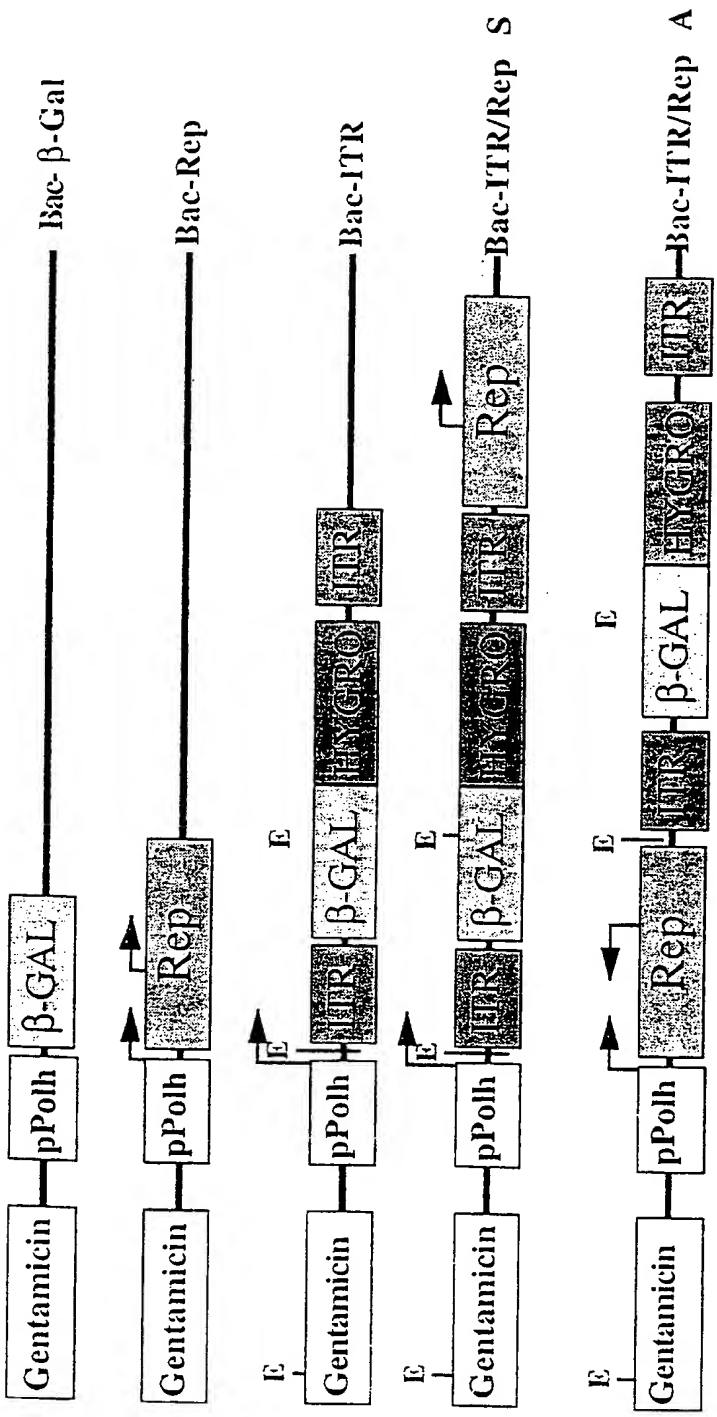


Fig. 6

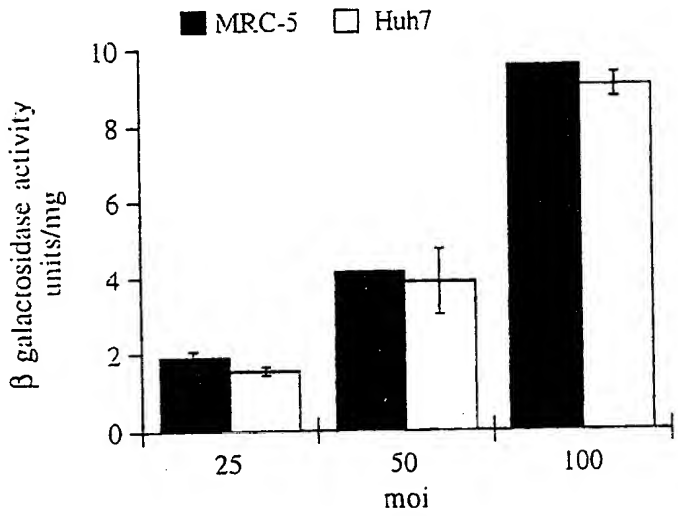
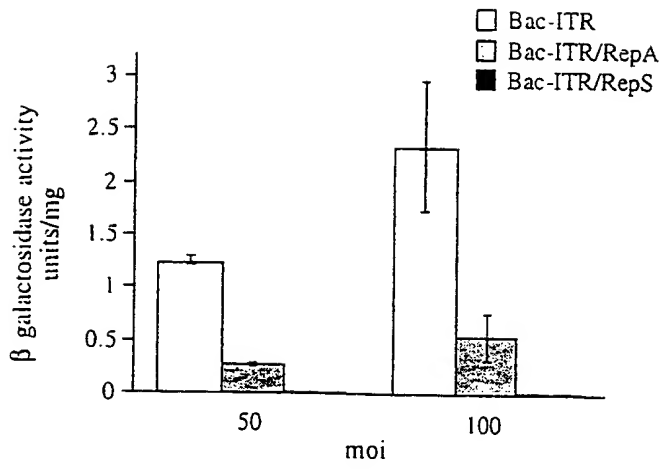

MRC-5
Huh7
β galactosidase activity units/mg
10
8
6
4
2
0
25
50
100
moi

Fig. 7


Bac-ITR
Bac-ITR/RepA
Bac-ITR/RepS
β galactosidase activity units/mg
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2.5
2
1.5
1
0.5
0
50
100
moi

Fig. 8

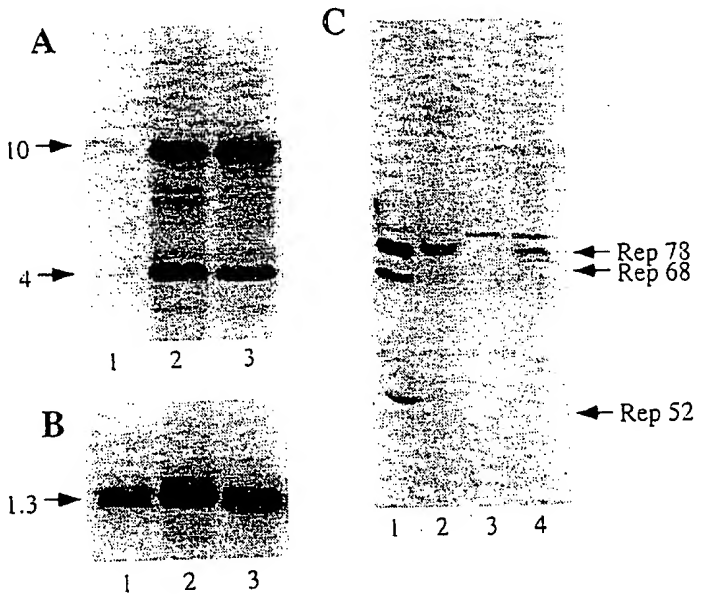


Fig. 9

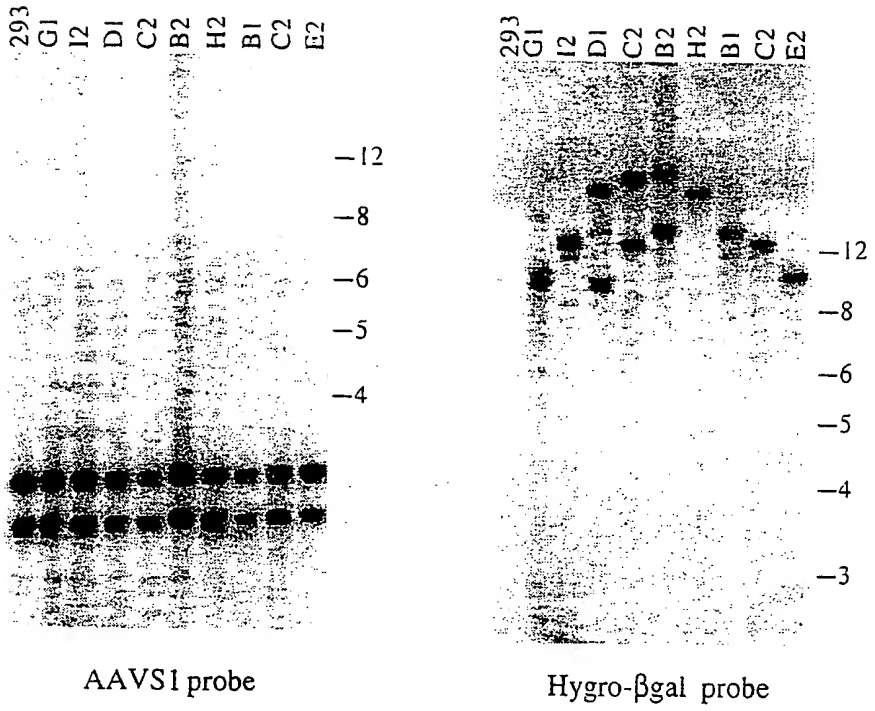
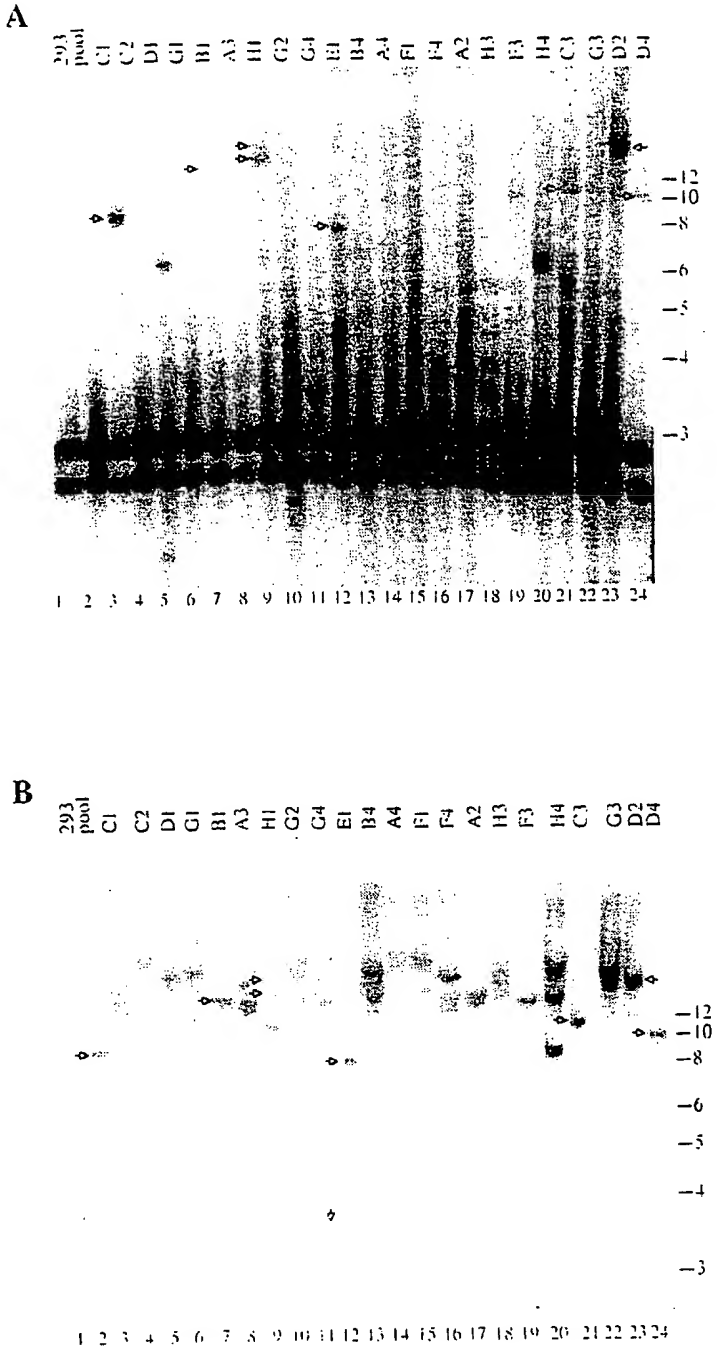


Fig. 10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12
10
8
6
5
4
3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
B
293 pool C1 C2 D1 G1 B1 A3 H1 G2 G4 E1 B4 A4 F1 F4 A2 H3 F3 H4 C3 G3 D2 D4
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10
8
6
5
4
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Fig.11

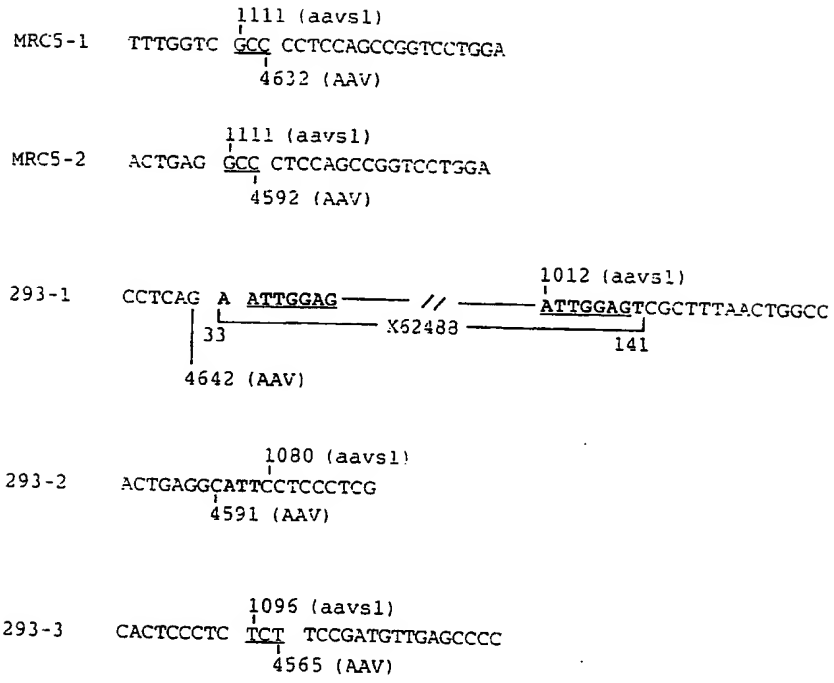
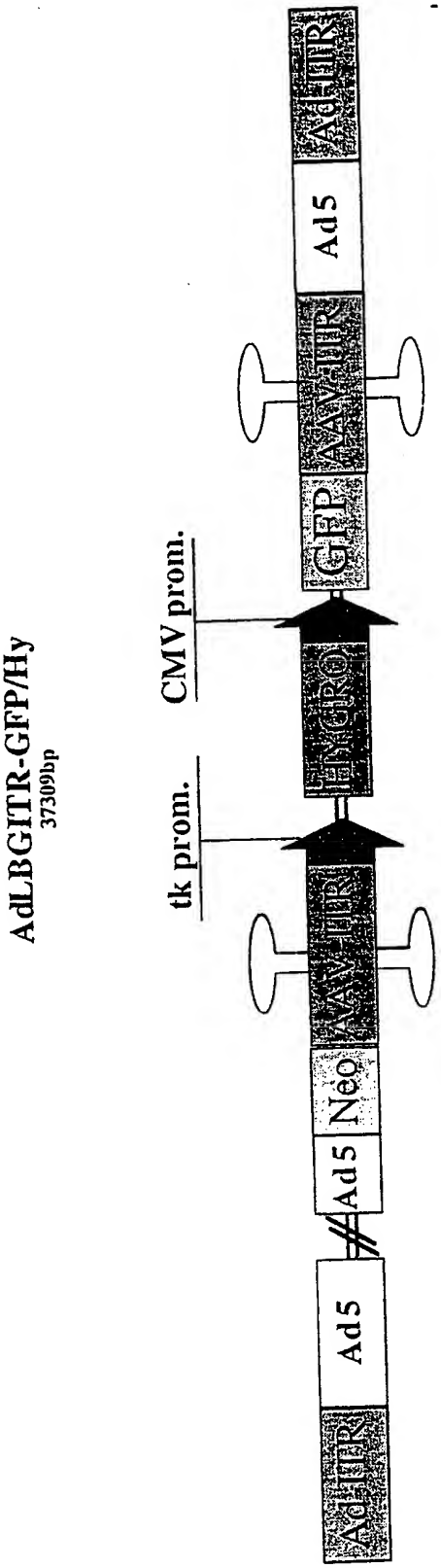


Fig. 12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37309bp
tk prom.
CMV prom.
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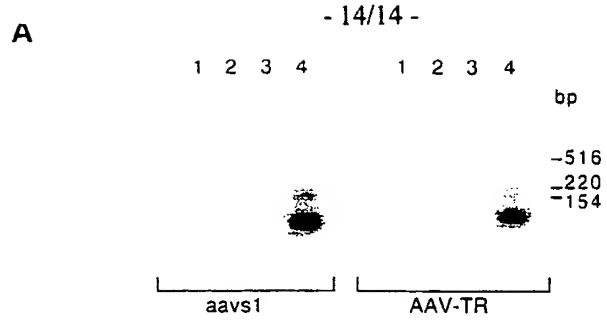
Ad5
Neo
Ad5
AAV ITR
ENGRO
GFP
AAV ITR
Ad5
Ad5 ITR

Fig. 13



B

HuH7

AAV-ITR 70 1158 aavs1

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A' C

HepG2

AAV-ITR 95 1294 aavs1

AAGGTCGCCC GACGCCCGGGCTTTcgtaac tgccg CTCTCCTGAG TCCGGACCAC TTTGAGCTTT

B C

Fig. 14

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/IT 98/00082

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/12 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 96 09074 A (GEN HOSPITAL CORP) 28 March 1996 see page 29, line 30 - page 30, line 20; figure 5 ---	1,2,5, 9-16 6-8
X Y	WO 96 18727 A (AVIGEN INC) 20 June 1996 see the whole document ---	1-4,9-16 5
Y	HOFMANN C. ET AL.: "Efficient gene tranfer into human hepatocytes by baculovirus vectors." PROC. NATL. ACAD. SCI. USA, vol. 92, October 1995, pages 10099-10103, XP002072661 cited in the application see the whole document --- -/--	5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 1998	12/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/IT 98/00082

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	JOHNSTON K. M. ET AL.: "HSV/AAV hybrid amplicon vectors extend transgene expression in human glioma cells." HUMAN GENE THERAPY, vol. 8, no. 3, 10 February 1997, pages 359-370, XP002072662 see the whole document	1,2,4, 9-16
X	WO 96 13598 A (UNIV PENNSYLVANIA ;WILSON JAMES M (US); KELLEY WILLIAM M (US); FIS) 9 May 1996 cited in the application see the whole document	1-3,9-16
X	WO 95 23867 A (RHONE POULENC RORER SA ;DENEFLE PATRICE (FR); LATTA MARTINE (FR);) 8 September 1995 see the whole document, especially page 12, lines 3-7	1-3,9-16
Y	BOULIKAS T. ET AL.: "HISTONES, PROTAMINE, AND POLYLYSINE BUT NOT POLY(E:K) ENHANCE TRANSFECTION EFFICIENCY" INTERNATIONAL JOURNAL OF ONCOLOGY, vol. 10, no. 2, February 1997, pages 317-322, XP002058322 see the whole document	6
Y	FELGNER P. L.: "PARTICULATE SYSTEMS AND POLYMERS FOR IN VITRO AND IN VIVO DELIVERY OF POLYNUCLEOTIDES" ADVANCED DRUG DELIVERY REVIEWS, vol. 5, no. 3, September 1990, pages 163-187, XP000603153 see page 179, last paragraph - page 181, last paragraph	7,8
T	PALOMBO F. ET AL.: "Site-specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector." JOURNAL OF VIROLOGY, vol. 72, no. 6, June 1998, pages 5025-5034, XP002072663 see the whole document	1-16

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/IT 98/00082

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